REMARKS/ARGUMENTS

Claims 38, 40-42, 62 and 75 are pending. Claims 38, 40 and 62 have been amended herein. Claims 1-37, 39 43-61 and 63-74 have been cancelled without intending to abandon or to dedicate to the public any patentable subject matter. As set forth more fully below, reconsideration and withdrawal of the Examiner's rejections of the claims are respectfully requested.

Objection to the Specification

The Examiner has objected to the specification as containing trademarks that are not capitalized. The Specification has been amended herein to overcome this objection.

The Examiner has also objected to the title of the invention. Applicants will change the title of the invention after the identification of any allowable subject matter.

Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected Claims 38-42 and 62 under 35 U.S.C. § 112, first paragraph, as lacking enablement for all protein variants, cytokine variants, and 4-alpha helix bundle cytokines comprising the substitution of valine for phenylalanine.

The Examiner has also rejected Claims 38-42 and 62 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

Specifically, the Examiner argues that while the specification is enabling for TPO muteins and EPO muteins, the assertion that all phenylalanine to valine substitution mutiens of all protein variants, cytokine variants and 4-alpha helix bundle cytokines will have biological activities similar to the TPO muteins and EPO muteins cannot be accepted in the absence of direct supporting evidence, which is not provided in the specification, because the relevant literature reports examples of polypeptide families wherein individual members have distinct and sometimes even opposite biological activities.

The binding affinity between a cytokine and the corresponding receptor depends upon the physical properties of the amino acids themselves, including their hydrophobic index and size. In accordance with the present invention, a cytokine variant which substitutes valine for phenylalanine residues in a binding domain will have enhanced physiological activity modulating effects for reasons related to the physical properties imparted by the amino acid substitution as follows:

1) Phenylalanine is a relatively nonpolar amino acid that has an aromatic side chain and a hydrophobicity index of 3.0. Valine is a nonpolar hydrophobic amino acid that has an aliphatic side chain and a hydrophobicity index of 4.0. Additionally, because valine is smaller than phenylalanine, a cytokine that has substitutions of valine for phenylalanine residues will fit deeper in the binding pocket of the corresponding receptor. Thus, a cytokine that substitutes valine for phenylalanine residues in the binding domain will have an increased hydrophobic force and will be positioned deeper in the receptor binding site leading to an increased binding affinity, leading to an increased biological response modulating efficiency.

2) The binding domains of proteins generally have a hydrophilic region at the NH-ward and COOH-ward sites of the hydrophobic region. In all cases, protein binding in the hydrophilic region occurs first, followed by binding in the inner, hydrophobic region. This process is true of all proteins, including cytokines. The substitution of valine for phenylalanine in the hydrophobic region readily produces a variant having a high binding affinity. Thus, the substitution of valine for phenylalanine within the hydrophobic core of binding domain changes cytokines, including 4-alpha helix bundle cytokines, into nonpathogenic mutants having higher binding affinities.

3) The substitution of valine for phenylalanine residues, as a conservative substitution, has minimal influence on the secondary or tertiary structure of a protein and thus rarely affects the function of the protein. Further, because phenylalanine is mainly present in a highly hydrophobic region, it is rarely exposed to the exterior of the protein in typical physiological fluids. When phenylalanine residues are replaced by valine, the modified protein becomes smaller and more tightly compressed in tertiary structure due to the higher hydrophobicity of

valine. For this reason, the valine-for-phenylalanine modified protein exhibits less potential to illicit an antibody reaction.

For these reasons, the enhanced receptor binding effects described in the present invention are achieved with any cytokine, including 4-alpha helix bundle cytokines, and the modified cytokines exhibit higher binding affinity and biological activity than wild type cytokines while avoiding the problems associated with conventional protein mutants, such as antibody production.

Evidence that a substitution of valine for phenylalanine residues leads to increased binding affinity is supported by the finding of FcγRIIIa (CD16) mutation expressed on NK cells in human autoimmune diseases as described by Jianming Wu et al. (Clinical Investigations, 100:1059-70 (1997), a copy of which is enclosed here for the Examiner's convenience). As described in this research, the human receptor protein has a genetic polymorphism such that two groups of individuals exist: one group has phenylalanine at position 176 of the receptor (a position recognized as participating in Fc recognition of an antibody ligand), while the other group has valine at this position. Individuals having phenylalanine at position 176 of the receptor exhibit weakened binding affinity for the FC region of the antibody ligand and are highly susceptible to systemic lupus erythematosus (SLE).

Further evidence is found in Tim Clackson et al. (PNAS USA 95:10437 (1998) a copy of which is enclosed with this response for the Examiner's convenience). This reference describes the affinity of intracellular proteins with a FKBP sequence in the binding domain for certain organic compounds. The authors show that the binding affinity of a modified FKBP protein having a truncated phenylalanine residue in the binding domain for a modified organic compound increases thousands of times over the affinity of the wild type protein for the wild type ligand. These data show that any proteins, including 4-alpha helix bundle cytokines, can have substantially increased binding affinity following minor changes to phenylalanine residues within the binding site.

The Examiner notes that the specific examples of nine different TPO and EPO muteins provided in the specification sufficiently describes the use of TPO and EPO cytokines in the methods of the present invention but argues that the direction or guidance presented in the specification is insufficient to support the production and use of all cytokine variants having a valine for phenylalanine substitution, and that one of skill in the art would be required to undertake undue experimentation to modify and test all cytokines of interest. It is the Examiner's position that while the required methods are well known and the skills of those of the relevant art are high, the skilled artisan could only carry out the claimed methods with the two proteins for which specific examples are provided. But Applicants submit that undue experimentation is not viewed solely in light of the examples provided. Indeed, as stated in the guidance provided by section 2164.02 of the MPEP:

> The presence of only one working example should never be the sole reason for rejecting claims as being broader than the enabling disclosure, even though it is a factor to be considered along with all the other factors. To make a valid rejection, one must evaluate all the facts and evidence and state why one would not expect to be able to extrapolate that one example across the entire scope of the claims.

In this instance, the Examiner is arguing that the reason the highly skilled artisan could not use the working examples provided to extrapolate to the entire scope of the claims is because the examples show differing activity within the TPO and EPO muteins produced and because there is a general art acknowledgment that knowledge of protein structure does not, by itself, predict protein function. Thus, the Examiner argues, it would take too much experimentation by the skilled artisan to make any modified protein and determine whether it is functional and, further that the specification does not teach the skilled artisan that Applicants had successfully practiced the invention and described the same at the time of filing the instant application.

But this is distinct from too much experimentation. The methodology used to arrive at the modified cytokines is well known, and the relevant skill in the art is high, and, as Applicants have described above, the outcome of enhanced receptor binding affinity is known and expected.

Thus, there is little experimentation necessary for one of skill in the art to carry out the known methods necessary to practice the presently claimed invention. However, Applicants agree with the Examiner that these known methods are labor intensive and require the skilled artisan to perform laboratory work to practice the invention. But this labor, and the work necessary, are not experimentation with an unknown outcome - they are merely the application of known methods by skilled artisans in which the outcome has been shown by working examples in the instant specification. Thus, the Examiner's review of the 'Wands' factors' confuses a labor intensive method with undue experimentation to conclude that the currently claimed methods are not enabled by the present specification. Applicants submit that too much work is not to be equated with undue experimentation and that the working examples and accompanying description provide adequate enablement for the currently claimed methods.

Claim Rejections Under 35 U.S.C. § 102

The Examiner has rejected Claim 38 under 35 U.S.C. § 102(b) as being anticipated by Smulevich et al., (Biochemistry 33(23):7398-7407 (1994)). Applicants have amended Claim 38 to restrict the claimed protein variant to a protein having a cytokine binding domain containing a valine for phenylalanine substitution within the binding domain. For this reason, Applicants submit that Smulevich et al. does not anticipate Claim 38, as amended. Applicants therefore respectfully request the Examiner's rejection under 35 U.S.C. § 102(b) be withdrawn.

Based upon the foregoing, Applicants believe that all pending claims are in condition for allowance and such disposition is respectfully requested. In the event that a telephone conversation would further prosecution and/or expedite allowance, the Examiner is invited to contact the undersigned.

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Biochemistry

Redesigning an FKBP–ligand interface to generate chemical dimerizers with novel specificity

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ABSTRACT **FKBP ligand homodimers can be used to activate signaling events inside cells and animals that have been engineered to express fusions between appropriate signaling domains and FKBP. However, use of these dimerizers *in vivo* is potentially limited by ligand binding to endogenous FKBP. We have designed ligands that bind specifically to a mutated FKBP over the wild-type protein by remodeling an FKBP-ligand interface to introduce a specificity binding pocket. A compound bearing an ethyl substituent in place of a carbonyl group exhibited sub-nanomolar affinity and 1,000-fold selectivity for a mutant FKBP with a compensating truncation of a phenylalanine residue. Structural and functional analysis of the new pocket showed that recognition is surprisingly relaxed, with the modified ligand only partially filling the engineered cavity. We incorporated the specificity pocket into a fusion protein containing FKBP and the intracellular domain of the Fas receptor. Cells expressing this modified chimeric protein potently underwent apoptosis in response to AP1903, a homodimer of the modified ligand, both in culture and when implanted into mice. Remodeled dimerizers such as AP1903 are ideal reagents for controlling the activities of cells that have been modified by gene therapy procedures, without interference from endogenous FKBP.**

A number of natural and synthetic compounds are known that bind with high affinity to the human protein FKBP12 (1–5). Bivalent versions of these compounds—chemical "dimerizers"—recently have been developed as cell-permeant reagents to control intracellular signaling events that are naturally regulated by protein–protein interactions (6, 7). In these applications, cells are engineered to express a chimeric protein comprising a signaling domain fused to one or more FKBPs; treatment with dimerizer crosslinks the proteins and initiates signaling. Because genes for such fusion proteins can be delivered via gene therapy strategies, dimerizers might be used to control a wide range of proliferation and differentiation events for therapeutic purposes (8, 9).

Use of FKBP dimerizers *in vivo* is complicated by their potential interactions with endogenous FKBPs, which are ubiquitous and highly expressed in mammals. These interactions could blunt potency by allowing nonproductive dimerization events and compound sequestration. High concentrations of ligand also might interfere with the physiological functions of FKBP12, such as calcium channel modulation and regulation of cardiac development (10–12). The ideal dimerizer therefore would interact minimally with endogenous FKBP; but any modifications that reduce binding would have equivalent effects on the affinity for the chimeric target protein.

We set out to solve this specificity problem by engineering a unique pocket into the target FKBP that differentiates it from the endogenous protein. This approach is feasible because dimerizer applications involve the delivery of the target as well as the drug. Our aim was to add "bumps" to ligands to block sterically binding to wild-type FKBP and then generate compensating "holes" by mutagenesis that could be installed into target fusion proteins. We report here the design of such a remodeled ligand–FKBP pair and evaluation of the resulting bumped dimerizer as a signaling reagent *in vivo*.

MATERIALS AND METHODS

Synthetic Chemistry. The synthesis of compound **1** has been described (13). Isomers of compound **5** were prepared as shown in Fig. 3. Compounds **2–4** were prepared analogously by coupling amine **7** (Fig. 3) with 3,3-dimethyl-2-methoxypentanoic acid, 3,3-dimethyl-2-ethyl-pentanoic acid, or 2-phenyl-butanoic acid, respectively. FK506 was coupled to 4′-(aminomethyl)-fluorescein (4′-AF; Molecular Probes) analogously to the synthesis of FK1012 (6). Fluoro-5*S* was prepared by coupling 5*S* to 4′-AF by using 1,3-dicyclohexylcarbodiimide and 4-dimethylaminopyridine in dichloromethane.

Protein Engineering and Expression. FKBP and its mutants were expressed with an N-terminal hexa-histidine purification sequence and an influenza hemaglutinin (HA) epitope tag. Expression vector pET-H6HA-FKBP was assembled by cloning an *Nde*I–*Nde*I oligonucleotide pair encoding the amino acid sequence MHHHHHHYPYDVPDYAAMAHM and an *Nde*I–*Bam*HI PCR product encoding human FKBP12 into *Nde*I/*Bam*HI-digested pET20b(+) (Novagen). Mutants were engineered as described (14). Proteins were expressed in *Escherichia coli* BL21(DE3) and released in ≥50% purity by a single freeze-thaw step as described (15) and were purified further on Ni-NTA agarose (Qiagen) under native conditions. For structural studies, an expression vector for untagged F36V-FKBP was generated by excising the *Nde*I fragment from pET-H6HA-F36V-FKBP followed by recircularization. Protein for crystallography was expressed and extracted as before and purified to homogeneity by DEAE ion-exchange chromatography followed by gel filtration on S-100 Sepharose.

FKBP Binding Assay. Fluorescence polarization competition assays were performed as described (C.T.R., E. Laborde,

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This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: FKBP, human FK506-binding protein 12; F36V-FKBP, FKBP mutant with Phe36 replaced by Val; hGH, human growth hormone.
Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, Biology Department, Brookhaven National Laboratory, Upton, NY 11973 (PDB ID code 1bl4).
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†Present address: MGH Cancer Center, 13th Street, Charlestown, MA 02129.
‡Present address: Harvard University School of Public Health, Huntington Avenue, Boston, MA 02115.

D.A.H., and T.C., unpublished work). In brief, subsaturating concentrations of protein were incubated for 30 min with 2.5 nM fluoro-FK506 and serial dilutions of competitor ligand in the wells of a Dynatech microfluor plate. Polarization was read on a Jolley FPM-2 (Jolley Consulting and Research, Grayslake, IL). The increase in polarization on binding was used as a direct readout of percentage of bound probe, compared with controls containing no competitor (100%) or no protein (0%). The concentration of competitor resulting in a 50% probe displacement (IC_{50}) was determined by a nonlinear least square fit by using the four-parameter algorithm

$$P = P_{min} + (P_{max} - P_{min})/1 + \exp(a(IC_{50} - [\text{competitor}]))$$

where P_{min}, P_{max}, and P represent the minimal, maximal, and measured polarization values, respectively. Direct binding experiments with fluoro-5*S* used 10 pM ligand, and polarization was read on a Jolley FPM-1 reader.

X-Ray Crystallography. Crystals of the complex of F36V-FKBP with 5*S* were obtained by vapor diffusion in hanging drops containing 40 mg/ml complex in 1.2 M ammonium sulfate, 0.1 M sodium phosphate (pH 6.0), and 20 mM DTT over reservoirs of 2.4 M ammonium sulfate and were triclinic (a = 32.73, b = 41.85, c = 43.59 Å, α = 62.61, β = 82.75, γ = 85.29°) with two complexes in the asymmetric unit. Data were collected at room temperature with a Rigaku R-AXIS II area detector with graphite monochromated Cu Kα x-rays. Data were collected as 2° oscillation images, reduced to integrated intensities with the program DENZO (16), and scaled with ROTAVATA, and AGROVATA (17). The structure was solved by molecular replacement with AMORE (17) and refined by using X-PLOR (18). The current R value for reflections with $F > 2\sigma$ from 10.0 to 1.9 Å is 0.21 and R_{free} is 0.26; rms deviations are 0.009 Å for bonds and 1.78° for angles. Coordinates have been deposited with the Brookhaven Protein Data Bank (PDB ID code 1bl4).

Cell Line for *in Vitro* Fas Studies. Construction of the retroviral plasmid pSRα-myristoylation-2xFKBP-Fas-E has been described (13). An equivalent vector incorporating the F36V mutation in both FKBPs was prepared analogously. Cloned HT1080 cell lines (ATCC CCL-121) retrovirally transduced with Fas constructs were prepared as described (13). Cell viability after overnight incubation with AP1903 was measured by Alamar Blue assay (13).

Cell Line for *in Vivo* Fas Studies. pSRα-LNGFR-2x(F36V-FKBP)-Fas-E encodes the extracellular and transmembrane portions (amino acids 1–274) of the human low affinity nerve growth factor receptor (19), derived by PCR from a human cerebellum cDNA library, in place of a myristoylation sequence. The constitutive human growth hormone (hGH) expression plasmid pCEP4-hGH was made by subcloning a genomic hGH clone (20) into the episomal plasmid pCEP4 (Invitrogen). To prepare cells that constitutively express both F36V-FKBP-Fas and hGH, a G418-selected population of HT1080 cells retrovirally transduced with pSRα-LNGFR-2FKBP F36V-Fas-E was transfected with pCEP4-hGH by lipofection, and clones were selected in hygromycin B. Clone HTFasGH-3 was selected for its high response to AP1903 in the absence of actinomycin D, and high hGH production.

Animal Experiments. Male *nu/nu* mice were obtained from Charles River Laboratories (Wilmington, MA). For injection, HTFasGH-3 cells were harvested from tissue culture dishes in PBS/0.1% glucose/10 mM EDTA, washed, and resuspended in PBS/0.1% BSA/0.1% glucose at a concentration of 2×10^7 cells/ml. Between 2 and 4×10^6 cells were implanted into two i.m. sites. After 24 h, mice were administered i.v. AP1903 formulated in [50% *N,N*-dimethylacetamide/50% (90% PEG-400/10% Tween 80)] at 2 ml/kg. After a further 24 h mice were killed and serum hGH concentrations were determined by ELISA (Boehringer Mannheim 1-585-878).

RESULTS

Molecular Design. The natural product FK506 and synthetic derivatives exemplified by compound **1** (Fig. 1*A*) share a conserved α-keto-pipecolylamide core that binds in a complementary hydrophobic pocket on FKBP, as revealed by x-ray crystallography (Fig. 1*B*) (2, 5, 21). We focused on the C9 carbonyl oxygen, which packs tightly against Tyr26, Phe36, and Phe99, all making unusual edge-on ε-hydrogen contacts. Inspection of crystal structures suggested that replacing this carbonyl with larger substituents would dramatically reduce FKBP binding through steric clashes with either Phe36 or Tyr26 (depending on the stereochemistry at a tetrahedral C9 carbon; Fig. 1*A*). This in turn suggested that mutations that truncate these side chains might provide compensatory holes that would restore binding.

Interface Remodelling. We engineered a set of FKBP mutants in which Phe36 was replaced with smaller hydrophobic residues and measured their affinities for C9-alkylated analogs of compound **1** by using a competitive fluorescence polarization assay (Table 1). This assay reports IC_{50} values that are proportional to dissociation constants (K_d). Compound **1** bound to wild-type FKBP with an IC_{50} of 86 nM [corresponding to a K_d of ≈20 nM (2)], and binding was largely unchanged for the Phe36 mutants. Even modest enlargements of the C9 substituent to methoxy (compound **2**) or ethyl (compound **3**) reduced affinity to undetectable levels (IC_{50} > 10,000 nM), as expected. However, binding could be rescued by any of the Phe36 mutations, and although affinities were fairly low, the compounds showed apparent selectivity of up to 50-fold for the mutants over wild type. Two further trends were apparent. First, compound **3** bound more tightly than **2**, indicating that nonpolar substituents may be preferred. Second, the mutants clearly favored the *S*-isomer of compounds over the *R*. This is the preference predicted by modeling, so it suggested that the modifications were acting as specificity determinants as envisaged by our design.

To try to improve the affinities of these specific pairings, we retained the ethyl bump of **3** and varied the *tert*-pentyl "bottom" C9 substituent (2, 4, 5). Installing a phenyl group (compound **4**) improved affinities for each of the mutants by 3- to 5-fold while maintaining the lack of detectable binding to wild-type protein, and additional 5- to 20-fold increases were observed when a trimethoxyphenyl group was incorporated (compound **5**). As the affinities improved, so did the preference for the *S*-isomer over *R*, suggesting that the changes were not altering the binding of the engineered bump. Strikingly, none of the bumped compounds showed any major preference for a particular mutant; instead, affinities were largely insensitive to the size of the new side chain. This implied that, rather than a precise "lock-and-key" fit, molecular recognition in the new pocket was rather loose and flexible.

The best combination was the pairing of 5*S* with the Phe->Val mutant (F36V-FKBP), with an IC_{50} (1.8 nM) close to the resolution limit of the assay. To assess the true affinity of the compound, we coupled it to fluorescein and performed direct fluorescence polarization titration. The labeled compound, fluoro-5*S*, had a K_d of 0.094 nM for the F36V mutant, compared with 67 nM for the wild-type protein (data not shown). Thus, although binding to the wild-type protein was still detectable, specificity for F36V-FKBP was almost three orders of magnitude. Moreover, the affinity of fluoro-5*S* for F36V-FKBP exceeded that of any known ligand for the wild-type protein (1, 5).

Crystallographic Analysis of Remodeled Interface. To determine the structural basis for these binding characteristics, we crystallized the complex between 5*S* and F36V-FKBP and determined its structure to 1.9-Å resolution (Fig. 2). Overall, except for the mutation, the structure of the protein is essentially superimposable with wild-type FKBPs from unmodified

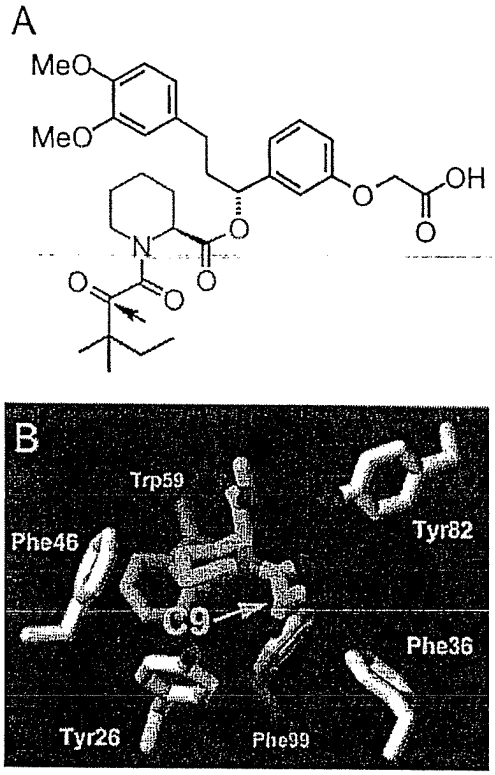


FIG. 1. (*A*) Chemical structure of synthetic FKBP ligand **1**, with the α-keto-pipecolylamide core region colored red. The arrow indicates the C9 carbonyl position modified in this study. A carboxylate group (*Right*) was included in all ligands to facilitate subsequent engineering of dimers. (*B*) Portion of the x-ray crystal structure of the complex between human FKBP12 and a synthetic compound related to **1** (from ref. 2). Only the α-keto-pipecolylamide core of the compound is shown, corresponding to the region colored red in Fig. 1*A*.

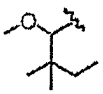
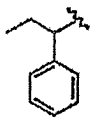
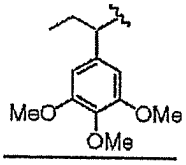
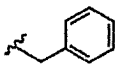
complexes (rms deviation for main chain atoms 0.45 Å). The F36V substitution opens up a cavity of ≈90 Å^3 (Fig. 2*A*), and surprisingly—apart from a 0.2-Å movement of the Val36 main chain—the protein does not relax to fill it. As a result, the pocket is lined by the predominantly hydrophobic side chains that usually pack against Phe36, and the Gly28 mainchain is exposed at the base of the pocket (Fig. 2*B*). The *S*-ethyl bump of the ligand inserts into this cavity exactly as predicted, penetrating what would be the surface of Phe36 in the wild-type protein and making multiple van der Waals contacts (Fig. 2 *C* and *D*). Compared with a complex between wild-type FKBP and a conventional ligand (2), the atoms of the core motif move by only 0.2–0.4 Å (Fig. 2*D*). Thus the modifications lead to a largely "surgical" deletion and insertion of contacts.

One remarkable consequence of this is that the bump only occupies ≈40% of the engineered hole, so that a large, fully enclosed cavity of ≈60 Å^3 remains within which no ordered water molecules are visible. Improving the fit by extending the ethyl bump of **5*S*** to an allyl group did increase affinity but only ≈3-fold (data not shown). Thus, affinity is largely insensitive to the quality of packing in the engineered pocket, possibly because recognition occurs mainly through nondirectional hydrophobic interactions (5). These structural observations correlate with our binding data showing that many Phe36 substitutions confer good binding affinity and that nonpolar bumps are preferred (Table 1).

The remaining peripheral substituents of **5*S*** pack into hydrophobic channels, essentially as observed for unbumped compounds (2). The conformations of these groups can vary widely between ligands (2, 4, 5), and because we do not have the structure of unbumped **5*S*** for direct comparison, we cannot assess effects of the modification—although the C9-trimethoxyphenyl group would have to shift because of the altered C9 stereochemistry (Fig. 2*D*). The substantial binding energy this group confers to **5*S*** (Table 1) probably stems from two good hydrogen bonds to Arg41 and His87 and also extensive intramolecular contacts (Fig. 2 *A* and *B*) that may preorganize the ligand and decrease the entropic cost of binding (2, 5).

Activation of Fas Signaling with a Remodeled Dimerizer. Having identified **5*S*** as an appropriately specific ligand, we coupled together two molecules through a previously identi-

Table 1. Binding affinities (IC_{50}s in nM) of unmodified and C9-bumped ligands **1**–**5** for a panel of FKBP Phe36 mutants

									
Position 36	**1**	**2*R***	**2*S***	**3*R***	**3*S***	**4*R***	**4*S***	**5*R***	**5*S***
Phe (wt) 	86	>10000	>10000	>10000	>10000	>10000	>10000	>10000	2930
Leu 	47	1300	850	1430	250	238	51	212	6.2
Ile 	43	2600	900	1300	190	346	93	163	5.1
Val 	44	3400	1120	1150	230	133	40	187	1.8
Ala 	174	7400	7400	580	400	460	66	431	18

For compounds 2–5, the affinities of the separated *R* and *S* isomers were determined. Only the lower portion of each compound is shown (see Fig. 1*A*), with the C9 carbonyl or bump substituents colored red. The high affinity interaction between compound **5*S*** and the Phe36Val mutant is boxed.

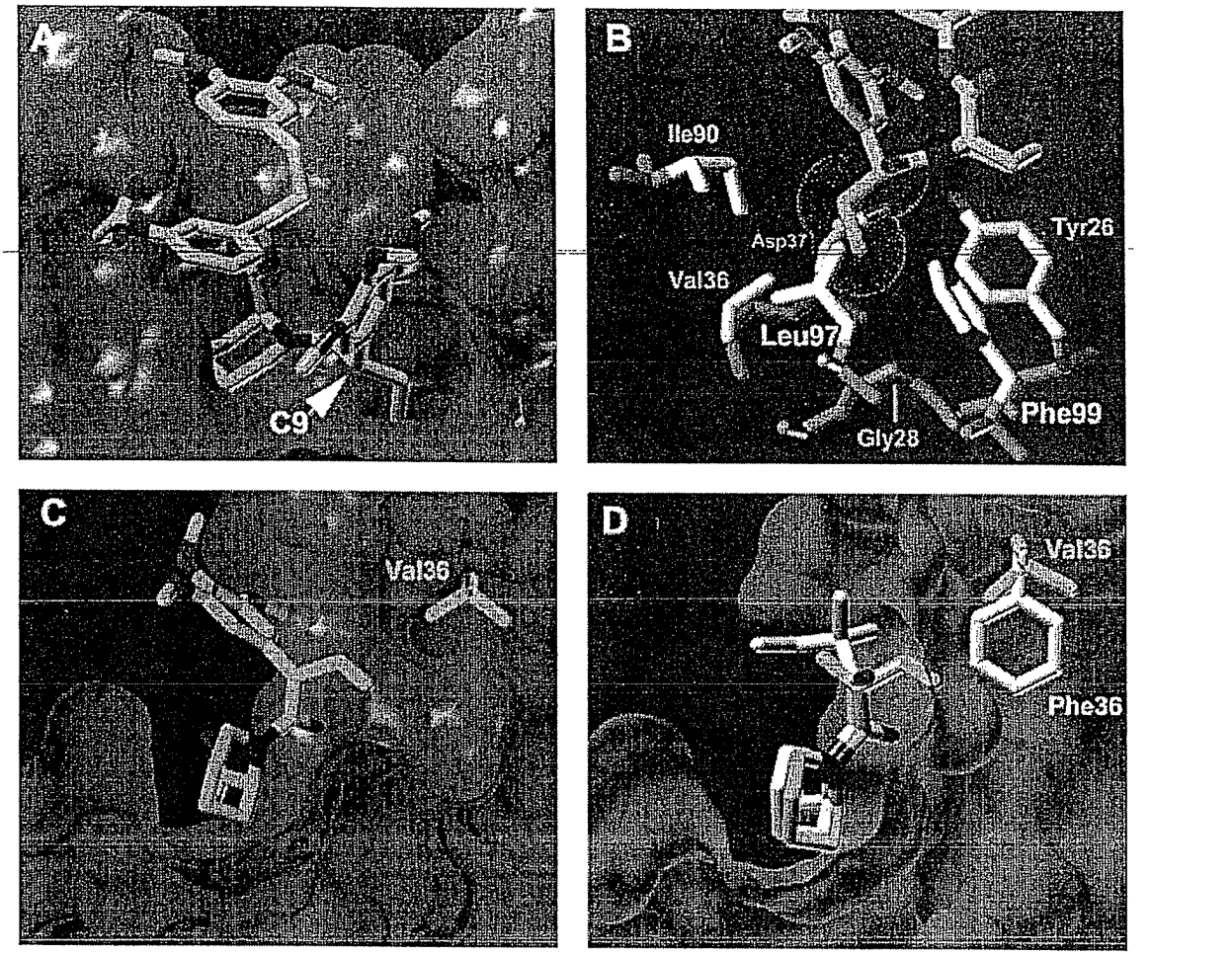


FIG. 2. X-ray crystal structure of the 5*S*-F36V-FKBP complex. (*A*) Overview of the complex. (*B*) Sidechains contacting the ethyl bump of 5*S*. Side chains are shown in gray, and main chain is shown in brown. The van der Waals surface of the ethyl bump of 5*S* is shown (yellow dots). (*C*) A section through the ligand binding site of the complex showing the large cavity created by the F36V mutation. (*D*) Equivalent section through the complex between the wild-type protein and an unbumped compound related to **1**, shown in white (2). For comparison, the Val36 side chain and the 5*S* core region from *C* are overlaid (yellow), based on a superposition of the main chain atoms in the two structures.

fied linker (13) to create the bumped dimerizer AP1903 (Fig. 3). The binding affinity and specificity characteristics of AP1903 mirrored those of its monomeric precursor (Fig. 4), confirming that AP1903 should interact minimally with endogenous FKBP inside cells. As a first test of the potency of AP1903, we determined its ability to activate signaling through a chimeric Fas receptor. Previous work showed that cells expressing membrane-tethered fusions between FKBP and the intracellular domain of the Fas receptor undergo apoptosis in response to appropriate dimeric FKBP ligands (8, 13). This approach is a promising general method for eliminating engineered cells following gene therapy procedures, but clinical application may require ligands that can operate independently of endogenous FKBP.

The human fibrosarcoma line HT1080 was engineered to express stably a fusion protein comprising a myristoylation sequence, two copies of F36V-FKBP, and the human Fas intracellular domain. AP1903 elicited potent and dose-dependent apoptotic death of these engineered cells in culture, with an EC_{50} of $\approx$0.1 nM (Fig. 5*A*). This potency corresponds directly to the binding affinity and represents a 60-fold improvement over the previous best unbumped compound (AP1510, a synthetic dimer of **1**; Fig. 1*B*) (13). By contrast, activity was reduced by over two orders of magnitude on an analogous cell line expressing a wild-type FKBP Fas fusion protein. Of interest, the level of discrimination (200-fold) was lower than that observed for binding *in vitro* (1,000-fold). We speculate that this reflects stabilization of the weak interactions between AP1903 and membrane-tethered tandem wild-type FKBP fusion proteins (the avidity effect). No effect was observed on unmodified HT1080 cells at concentrations up to 1 μM (data not shown). Thus, the affinity and specificity of 5*S* were reflected in dimerizer potency in cells.

Activation of Fas Signaling *in Vivo* by AP1903. To test whether AP1903 can function *in vivo*, we determined its efficacy in an mouse model of conditional cell ablation. We engineered a second HT1080 cell line expressing a Fas-F36V-FKBP construct that also constitutively secretes hGH. Constitutive hGH secretion provides a convenient and accurate way to monitor the number of viable cells *in vivo* because hGH has a serum half-life of only $\approx$3 min in mice (22, 23). Over 3 consecutive days, we implanted these cells i.m. into nude mice, treated the animals i.v. with various doses of AP1903, and then determined serum hGH levels as a measure of the number of surviving cells. Fig. 5*B* shows that AP1903 elicited a dose-dependent decrease in serum hGH levels, with a half-maximal effective dose of 0.4 $\pm$ 0.1 mg/kg. Thus, the bumped dimerizer retains potent activity *in vivo*, indicating that the remodeled interface is functional in a whole animal context.

DISCUSSION

General Implications for Drug Design. In this study, we redesigned a molecular interface to generate compounds that

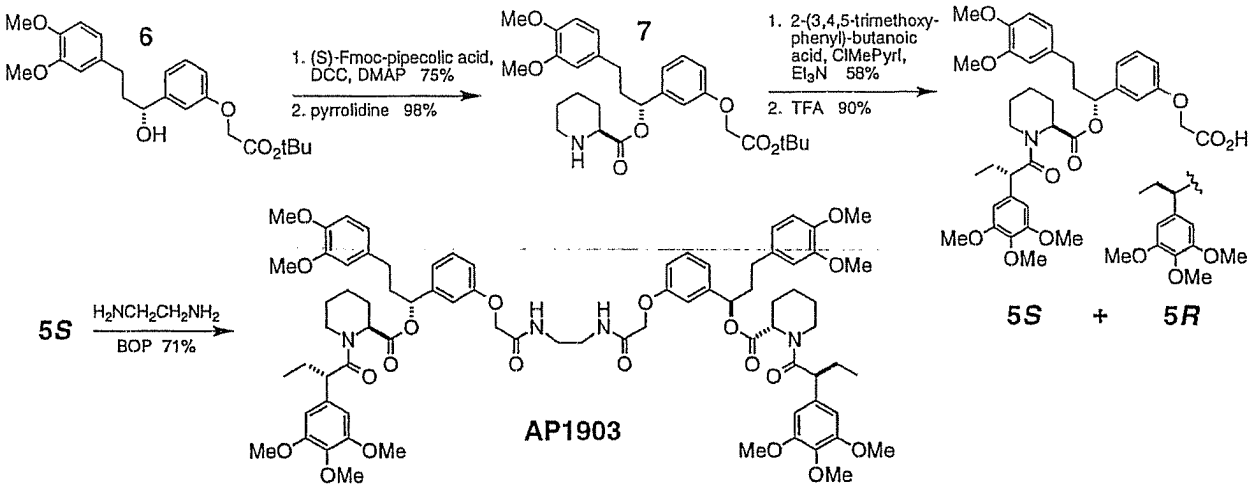


FIG. 3. Chemical structure of the C9-bumped dimerizer AP1903 and scheme for its synthesis via compound 5*S*. Preparation of alcohol 6 has been described (13). *R* and *S* isomers of compound 5 were chromatographically separated as *tert*-butyl esters before final TFA deprotection. Fmoc, *N*-(9-fluorenylmethoxycarbonyl); DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; ClMePyrI, 2-chloro-1-methylpyridinium iodide; TFA, trifluoroacetic acid; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; Me, methyl; Et, ethyl; iPr, isopropyl; *t*Bu, *tert*-butyl.

can differentiate 1,000-fold between two proteins differing by a single point mutation. This problem resembles a common challenge in conventional drug discovery, where efficacy can depend on discrimination between closely related homologs. For example, anti-inflammatory drugs that specifically inhibit human cyclooxygenase-2 (COX-2) while sparing COX-1 are expected to show significantly reduced toxicity (24). A cavity caused by the amino acid substitution Ile523Val is the major differentiating feature of the COX-2 binding site (25). Therefore, lessons learned in our work may be generally relevant to drug design.

In particular, we found that our designed ligand has subnanomolar affinity for its target despite the presence of an enclosed hole in the binding interface. Cavities at interfaces previously have been considered incompatible with high affinity ligand binding, which is invariably characterized by excellent shape complementarity (5). Furthermore, holes engineered into protein cores are destabilizing (26). Our observations suggest surprisingly that such holes need not be detrimental to affinity. Selectivity of conventional drugs might therefore be attained by relatively imprecise recognition of surface cavities in target proteins—particularly when those cavities are largely nonpolar—and binding affinity then improved by secondary modifications, as in this work.

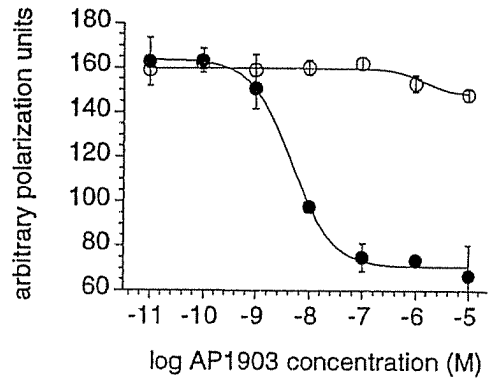


FIG. 4. Binding affinity and specificity of AP1903 determined by competition fluorescence polarization assay. Fluoresceinated FK506 probe was bound to wild-type FKBP (open circles) or F36V-FKBP (closed circles), and serial dilutions of AP1903 were added. AP1903 displaced the probe from F36V-FKBP with an IC_{50} of 5 nM, but binding to the wild-type protein was negligible.

Comparison to Other Remodeled Interfaces. We found that our interface modifications caused only local structural

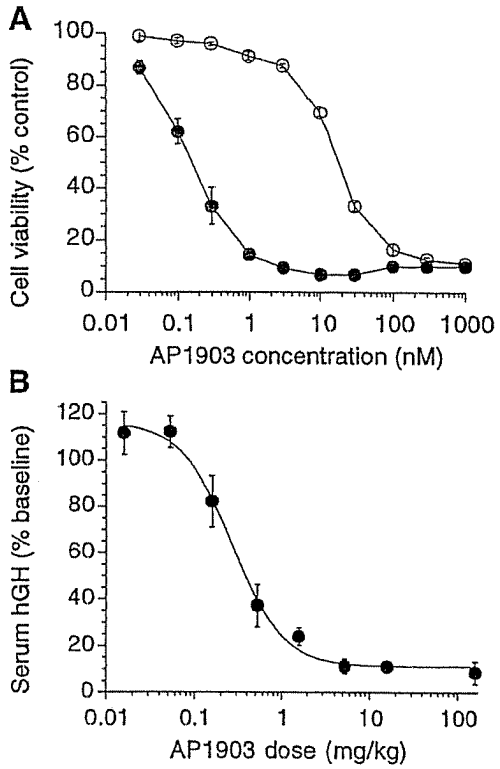


FIG. 5. Activation of Fas signaling by AP1903 *in vitro* and *in vivo*. (*A*) AP1903-induced killing of cells in culture expressing dimerizer-dependent Fas constructs. HT1080 cells stably transduced with retrovirus pSRα-myr-2FKBP-Fas-E (open circles) or pSRα-myr-2(F36V-FKBP)-Fas-E (closed circles) were treated overnight with the concentrations of AP1903 shown, and viability then was measured by Alamar Blue assay. Values shown are the means of triplicate wells. (*B*) AP1903-dependent elimination of hGH-secreting HTFasGH-3 cells implanted into nude mice. Serum hGH levels directly reflect the number of viable cells (see text). Values (mean ± 1 SEM) are from three to six separate experiments (at least three mice per point per experiment).

changes. Redesigned interfaces also have been engineered between cyclophilin and cyclosporin (27, 28) and between Src tyrosine kinase and ATP (29). Of interest, molecular modelling generally suggests that, in these cases, holes would remain unless rearrangements occurred (30), but no direct structural data are yet available. However, our results are in striking contrast to a recent report of a remodeled complex between two proteins, where major structural adjustments were propagated across the entire interface (31). Additional studies will be required to determine whether this reflects an inherent difference in "plasticity" between the two types of complex.

Future Applications of AP1903. By engineering out binding to endogenous FKBP, we have eliminated an important potential obstacle to the therapeutic use of chemical dimerizers. Unexpectedly, our design efforts also produced a ligand with higher affinity than any previous dimerizer. AP1903 should therefore be effective for controlling a wide range of cellular events inside cells and whole organisms, at low concentrations and independently of endogenous FKBP.

Inducible apoptosis may be an important early clinical application because the ability to eliminate engineered cells could be broadly useful—for example, to abort a graft-versus-host response after allogeneic bone marrow transplantation (32) or as a general failsafe for gene therapy procedures. Current approaches involve expressing nonhuman enzymes that can metabolize pro-drugs to lethal products (for example, the combination of herpesvirus thymidine kinase and gancyclovir), but immune recognition of the foreign protein is a serious problem (32, 33). AP1903-inducible Fas activation is an attractive alternative because apoptosis is a cell-autonomous and noninflammatory process that occurs rapidly and in the absence of cell division, and Fas-FKBP-F36V is built from human proteins and should be minimally immunogenic.

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A Novel Polymorphism of FcγRIIIa (CD16) Alters Receptor Function and Predisposes to Autoimmune Disease

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Abstract

A novel polymorphism in the extracellular domain 2 (EC2) of FcγRIIIA affects ligand binding by natural killer (NK) cells and monocytes from genotyped homozygous normal donors independently of receptor expression. The nonconservative T to G substitution at nucleotide 559 predicts a change of phenylalanine (F) to valine (V) at amino acid position 176. Compared with F/F homozygotes, FcγRIIIa expressed on NK cells and monocytes in V/V homozygotes bound more IgG1 and IgG3 despite identical levels of receptor expression. In response to a standard aggregated human IgG stimulus, FcγRIIIa engagement on NK cells from V/V (high-binding) homozygotes led to a larger rise in $[Ca^{2+}]_i$, a greater level of NK cell activation, and a more rapid induction of activation-induced cell death (by apoptosis). Investigation of an independently phenotyped normal cohort revealed that all donors with a low binding phenotype are F/F homozygotes, while all phenotypic high binding donors have at least one V allele. Initial analysis of 200 patients with SLE indicates a strong association of the low binding phenotype with disease, especially in patients with nephritis who have an underrepresentation of the homozygous high binding phenotype. Thus, the FcγRIIIa polymorphism at residue 176 appears to impact directly on human biology, an effect which may extend beyond autoimmune disease characterized by immune complexes to host defense mechanisms. (*J. Clin. Invest.* 1997. 100:1059–1070.) Key words: receptors, Fc • polymorphism, genetics • macrophages • killer cells, natural • lupus erythematosus, systemic

Introduction

Genetic polymorphisms of human FcγRIIa and FcγRIIIb have been characterized (1–13) and associated with certain disease risks (14–20). The two allelic forms of FcγRIIa differ by two nucleotides (nt),[1] one in the first extracellular Ig-like domain (EC1) predicting a glutamine (Q) to tryptophan (W) at residue position 27 and one in the second extracellular Ig-like domain (EC2) predicting an arginine (R) to histidine (H) at residue position 131. The change at position 131 markedly alters the ability of the receptor to bind human IgG2 (10, 12), and this polymorphism has been associated with certain bacterial infections (14, 15, 19) and with SLE (16–18). The two allelic forms of neutrophil-specific FcγRIIIb differ by five nucleotides which results in four amino acid differences in EC1 (21). Although binding of IgG does not seem to be affected (9), these two allelic forms do have different levels of quantitative function (9, 10), and the more active NA1 allele has been associated with severe renal disease in certain systemic vasculitides (20).

Several recent observations suggest that FcγRIIIa, which is expressed on natural killer (NK) cells, mononuclear phagocytes, and renal mesangial cells (22), might also be polymorphic in both its structure and quantitative expression. Vance and Guyre originally described a functional polymorphism in FcγRIIIa on NK cells among normal donors (23). Based on some differences both in IgG binding and in anti-CD16 reactivity, they suggested that variations in receptor expression might explain their observations. More recently, de Haas and colleagues have described a triallelic sequence polymorphism at nt 230 in FcγRIIIA (24). This single nucleotide substitution in the third exon encoding EC1 predicts an amino acid change from leucine (L) to arginine (R) or from leucine (L) to histidine (H) and reportedly influences the binding of human IgG and several anti-CD16 mAbs (24, 25). Such structural variants of FcγRIIIa, recognized by altered patterns of anti-CD16 mAb binding, may be related to a clinical phenotype of repeated infections (26).

The FcγRIIIA sequence polymorphism on NK cells which reportedly influences ligand binding (24) raised the possibility that this sequence polymorphism might explain previously described differences in NK FcγRIIIa and NK cell function (23, 26). To test this hypothesis, we identified several of the normal donors studied by Vance and Guyre (23) and characterized the nucleotide sequence of their FcγRIIIA. Contrary to our expectation, these donors were monomorphic at nt 230 and nt 248 (amino acid positions 66 and 72).[2] However, they were polymorphic at nt 559, a site noted by Ravetch and Perussia as

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1. *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; EC, extracellular domain; GAM, goat anti-mouse IgG; MNC, mononuclear cells; NK, natural killer; nt, nucleotide; PE, phycoerythrin; PI, propidium iodide; TC, tri-color.

2. The first amino acid of the signal sequence is designated amino acid 1 (21). In this nomenclature, nt 559, 230, and 248 are in the codons for amino acids 176, 66, and 72, respectively. Using the first amino acid of EC1, others have designated nt 230 and 248 polymorphisms as amino acids 48 and 64.

potentially polymorphic (21). This nonconservative T to G substitution predicts a change of phenylalanine (F) into valine (V) at position 176 in the membrane-proximal EC2. Since several studies suggest that the second Ig-like domain strongly influences ligand binding (27–32), we pursued further characterization of this 176F/V polymorphism by identifying normal donors homozygous at position 176 (and homozygous at positions 66 and 72). Compared with F/F homozygotes, FcγRIIIa expressed in V/V homozygotes bound more IgG1 and IgG3 despite identical levels of receptor expression. These observations indicate that the sequence polymorphism at nt position 559 alters the apparent affinity of FcγRIIIa on both NK cells and monocytes for IgG. This difference affects the ability of the receptor to initiate a range of cell programs in response to a standard stimulus and underlies the previously described variation in NK FcγRIIIa function (23). Initial analysis of 200 patients with SLE indicates a strong association of the low binding phenotype with disease, especially nephritis, and a corresponding underrepresentation of the homozygous high binding phenotype. Thus, this polymorphism appears to impact directly on human biology, an effect which may well extend beyond autoimmune disease characterized by circulating immune complexes.

Methods

Donors. Anticoagulated peripheral blood was obtained from healthy normal volunteers and from 200 patients fulfilling the revised criteria of the American College of Rheumatology for SLE (33). All studies were reviewed and approved by each Institutional Review Board and all donors provided written informed consent.

Reagents. Human IgG (hIgG) subclass proteins were obtained from The Binding Site (San Diego, CA) or Sigma Chemical Co. (St. Louis, MO). All mAbs used were murine origin. Anti–human CD56-phycoerythrin (PE), anti–human CD14-tri-color (TC), anti–human CD3-FITC, anti-CD25-FITC, and anti-CD33-FITC were from Caltag Laboratories (Burlingame, CA). Anti-FcγRI (mAb 197, mIgG2a and mAb 22.2, mIgG1), anti-FcγRII (mAb IV.3, mIgG2b), and anti-FcγRIII (mAb 3G8-FITC, mIgG1) were from Medarex Inc. (Annandale, NJ). Other anti-FcγRIII mAbs used in this study were CLBFc Rgran1 (mIgG2a), B73.1 (mIgG1), 1D3 (mIgM), MEM154 (mIgG1), 30.2 (mIgG1), 214.1 (mIgG1), 135.9 (mIgG1), GRM1 (mIgG2a), and Leu11a (mIgG1). 1D3, MEM154, and CLBFcRgran1 were obtained through the 5th Leukocyte Typing Workshop. mAbs 30.2, 214.1, and 135.9 were generously provided by Dr. Howard Fleit (SUNY, Stony Brook, NY) (34); mAb GRM1 was from Research Diagnostics Inc. (Flanders, NJ). B73.1 (Leu11a) and Leu11a-FITC were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). FITC-conjugated and unconjugated goat anti–mouse IgG(H + L) (GAM), which recognizes mIgG1, IgG2a, and mIgM, was obtained from Boehringer Mannheim (Indianapolis, IN) and Jackson ImmunoResearch (West Grove, PA). Heat-aggregated human IgG was prepared by incubating the hIgG (Sigma), 20 mg/ml, at 63°C for 20 min.

Mononuclear cell (MNC) and NK cell preparation. Fresh anticoagulated blood was diluted 1:1 in Hanks' buffer (GIBCO BRL, Gaithersburg, MD) and centrifuged through a discontinuous two-step Ficoll-Hypaque gradient in 50-ml conical tubes (35). MNC were harvested from the upper and neutrophils from the lower Ficoll-Hypaque interface and washed three times with PBS, pH 7.4. After the last wash, the cells were resuspended, counted, and used either for mRNA preparations, for quantitative flow cytometry, or for further purification of NK cells.

NK cells were purified with the NK Cell Isolation Kit (Miltenyi Biotec Inc., Auburn, CA) which depletes human T cells, B cells, and myeloid cells from MNC by magnetic separation. Isolated NK cells were washed with Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4, and assayed for purity by flow cytometry with either the combination of anti-CD3-FITC, anti-CD14-TC, and anti-CD56-PE or the combination of 3G8-FITC, anti-CD14-TC, and anti-CD56-PE. Starting with 5×10^7 MNC, the yield of NK was typically $4–5 \times 10^6$ total cells with a purity of > 85%. The efficiency of NK cell recovery was ~ 75%.

Nucleic acid isolation. Total RNA was isolated from 10^7 MNC by using TRIzol™ total RNA isolation reagent (GIBCO BRL). 5 μg of total MNC RNA was used to synthesize cDNA with the SuperScript™ preamplification system (GIBCO BRL). For sequencing of genomic DNA and for allele-specific PCR, genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

RT-PCR and cDNA sequencing. To facilitate heterozygote detection, a dye primer strategy was used for fluorescence-based automated cycle sequencing of PCR product on an ABI 377 (ABI PRISM™ Dye Primer Cycle Sequencing -21M13 FS and M13REV FS Ready Reaction Kits; Applied Biosystems, Inc., Foster City, CA). Two overlapping sets of primers, with either M13 universal or reverse primer sequences at the appropriate 5′ ends, were designed for the FcγRIIIA cDNA. Sequencing set 4 (Fig. 1) was used to amplify position 50 to position 414: forward 5′-CAG GAA ACA GCT ATG ACC TCC CAA CTG CTC TGC TAC TT-3′ and reverse 5′-TGT AAA ACG ACG GCC AGT CCT CAG GTG AAT AGG GTC TTC-3′. Sequencing set 3 (Fig. 1) was used to amplify position 328 to position 869: forward 5′-TGT AAA ACG ACG GCC AGT CCG GTG CAG CTA GAA GTC CA-3′ and reverse 5′-CAG GAA ACA GCT ATG ACC GGG GTT GCA AAT CCA GAG AA-3′. The PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN Inc., Chatsworth, CA).

Allele-specific PCR. Three primers were designed for allele-specific PCR for genotyping genomic DNA at position 559 in FcγRIIIA (PCR set 1, Fig. 1 *A*). The FcγRIIIA-specific forward primer (5′-TCA CAT ATT TAC AGA ATG GCA ATG G-3′) corresponds to the FcγRIIIA sequence between position 449 and position 473 and was used in both T allele–specific and G allele–specific PCR assays. The reverse primers, corresponding to nt 586–559, provided allele specificity. The nt 559 G-specific reverse primer (5′-TCT CTG AAG ACA CAT TTC TAC TCC CTA C-3′) differs in one nucleotide from T-specific reverse primer (5′-TCT CTG AAG ACA CAT TTC TAC TCC CTA A-3′) at the 3′ end. The allele-specific PCR product of 138 bp was assayed on a 3% agarose gel. The PCR reaction was performed in a GeneAmp 2400 PCR System with 360 ng of DNA, 200 nM of each primer, 200 μM of dNTPs, 1.5 mM of MgCl, and 2.5 U of *Taq* polymerase (Boehringer-Mannheim Biochemicals) in a 50-μl reaction volume starting with 95°C for 5 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 72°C for 20 s with a final extension at 72°C for 7 min. The appearance of the 138-bp PCR products in the T or G allele–specific reaction indicates the presence of that allele.

Genomic DNA sequencing. To confirm FcγRIIIA genomic sequence, primers were designed to amplify a portion of exon 4 of FcγRIIIA which corresponds to EC2 (sequencing set 1, Fig. 1 *A*). The forward primer (5′-TGT AAA ACG ACG GCC AGT TCA TCA TAA TTC TGT CTT CT-3′, corresponding to nt 486–505) includes an intentional mismatch six nucleotides from the 3′ end to provide FcγRIIIA-specific priming. The reverse primer (5′-CAG GAA ACA GCT ATG ACC CTT GAG TGA TGG TGA TGT TCA-3′) corresponds to nt 610–590. The 162-bp PCR product containing the nt 559 polymorphic site was purified from a 3% agarose gel with the QIAquick Gel Extraction Kit. Fluorescence-based automated cycle sequencing of PCR product was performed on an ABI 377 (ABI PRISM™ Dye Primer Cycle Sequencing -21M13 FS and M13REV FS Ready Reaction Kits).

To determine the FcγRIIIA genomic sequence of EC1, primers were designed to amplify a portion of exon 3 encompassing nt positions 230 and 248 (sequencing set 2, Fig. 1 *A*). The forward primer (5′-CAG GAA ACA GCT ATG ACC CTC TTT CTG TAG CTT GGT TC-3′) anneals to the intron region between S1 and S2 of the

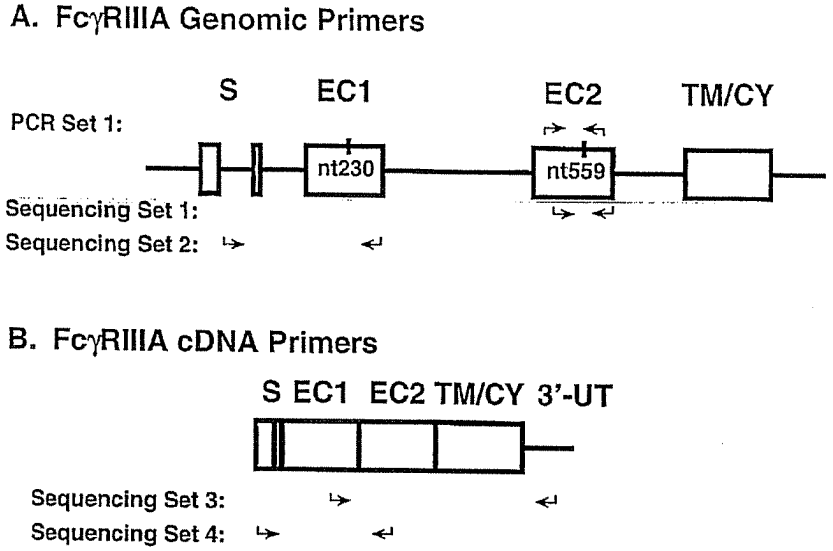

Figure 1. Schematic representation of the FcγRIIIA genomic structure and cDNA structure showing the relative location of primers used for PCR-based sequencing (sequencing sets 1–4) and allele-specific PCR (PCR set 1). For sequencing analysis, an M13-based dye-primer sequencing strategy was used. FcγRIIIa encoding cDNA was prepared from purified MNC. Relative positions of nt 230 and 559 are shown. *S*, Signal sequence (encoded in two exons); *TM/CY*, transmembrane/cytoplasmic domains; *3'-UT*, 3' untranslated sequence.

FcγRIIIA gene. The reverse primer (5'-TGT AAA ACG ACG GCC AGT ATG GAC TTC TAG CTG CAC-3') corresponds to nt 348 to 331 in exon 3. The PCR product was purified and sequenced as described above.

Flow cytometric assay for human IgG binding. Human IgG binding assays were performed using anticoagulated, washed whole blood. Mouse mAbs were used either for direct immunofluorescence (Leu11a-FITC, 3G8-FITC) or for indirect immunofluorescence (3G8, Gran1, B73.1, 1D3, MEM154, 30.2, 214.1, 135.9, GRM1) in conjunction with FITC-labeled $F(ab')_2$ GAM. Human IgG subclass myeloma proteins (IgG1, IgG2, IgG3, and IgG4) were directly conjugated with FITC according to standard techniques (36). Aggregates were removed by ultracentrifugation and removal was confirmed by the lack of binding of the FITC-labeled myelomas to human neutrophils. Before each experiment, the flow cytometer (FACScan®; Becton-Dickinson Immunocytometry) was calibrated with quantitative fluorescein microbeads (Flow Cytometry Standards Corp., Research Triangle Park, NC). Identification of individual cell populations was based on forward and right angle light scattering in combination with three-color immunofluorescence using TC, PE, and FITC.

For each IgG binding assay, 3 ml of heparinized whole blood was washed and cytophilic IgG was removed by incubation in 45 ml of PBS at 37°C for 20 min. For direct immunofluorescence, 100-μl aliquots of the washed whole blood were incubated at 4°C for 1 h with an hIgG-FITC myeloma protein (final concentrations of 15 and 30 μg/ml) or with anti-CD16 mAb [either directly FITC-conjugated or indirectly with $F(ab')_2$ GAM IgG-FITC, see below] and with CD14-TC and CD56-PE at 5 μg/ml. NK cells were identified as CD56-PE positive, CD14-TC negative cells within the lymphocyte light scatter gate; the binding of the different human IgG subclasses or the different anti-CD16 mAbs was assessed by the intensity of FITC fluorescence. Blood monocytes were identified as CD14-TC positive, CD56-negative cells within the typical blood monocyte light scatter gate. For myeloma protein binding to monocyte FcγRIIIa, washed whole blood was preincubated at 4°C for 10 min with mAb 197 IgG (10 μg/ml) to block the ligand binding site of FcγRI (12).

For the anti-CD16 mAb panel assay, washed whole blood cells were incubated with a saturating concentration of primary mAb for 30 min, washed twice with PBS, incubated with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG at 4°C for 30 min, and washed twice with PBS. After blocking remaining GAM binding sites with control mIgG1 and mIgG2a (10 μg/ml final concentration), CD14-TC and CD56-PE were added for phenotypic identification. After further incubation and washes, cells were analyzed on the FACScan®.

Measurement of change in $[Ca^{2+}]_i$. Changes in intracellular $[Ca^{2+}]_i$, induced by cross-linking of purified NK cell FcγRIIIa with heat-aggregated human IgG or with anti-FcγRIIIa mAb, were determined in purified indo-1-AM–loaded NK cells using an SLM 8000 spectrofluorometer and the simultaneous 405/490 nm fluorescence emission ratio as described previously (37, 38). Briefly, cells in suspension at 10^7 cells/ml in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4, were incubated with 5 μM indo-1-AM at 37°C for 15 min and washed in PBS. Cell preparations to be opsonized with mAb 3G8 were resuspended in Ca^{2+}- and Mg^{2+}-free PBS at 10^7 cells/ml, incubated with saturating concentrations of 3G8 (10 μg/ml) at 37°C for 5 min, and washed in PBS. All cells were resuspended in 1.1 mM Ca^{2+}, 1.6 mM Mg^{2+} PBS at 37°C for 5 min and then immediately transferred to a continuously stirring cell cuvette maintained at 37°C in the SLM 8000. With excitation at 355 nm, the simultaneous fluorescence emission at 405 and 490 nm was measured, integrated, and recorded each second. After establishing a base line for 60 s, either aggregated human IgG or goat $F(ab')_2$ anti–mouse IgG was added at final concentrations of 20 or 35 μg/ml, respectively, and data acquisition was continued for an additional 3.5 min. Each sample was individually calibrated by lysing cells in 1% Triton X-100 to determine the maximal emission ratio and by adding EDTA (20 mM final concentration) to determine the minimal ratio. The indo-1 fluorescence emission ratio was converted to $[Ca^{2+}]_i$ by the method of Grynkiewicz (39).

Induction of NK cell IL-2 receptor (CD25) expression and cell viability. Purified NK cells were cultured in RPMI/10% FCS and rIL-2 (100 U/ml) with or without a defined stimulus for varying periods of time in 96-well plates. FcγRIIIa-mediated stimulation of NK cells was effected either with the anti-FcγRIIIa mAb 3G8 IgG (10 μg/ml) or with heat-aggregated human IgG (20 μg/ml). For mAb stimulation, wells were precoated with 10 μg/ml $F(ab')_2$ GAM (Jackson ImmunoResearch) for 2 h at 37°C, rinsed, and 10^5 NK cells (10^6 cells/ml) were added with mAb and incubated for various periods of time. Aggregated human IgG was used either in solution as a soluble stimulus or immobilized to the tissue culture plated (precoating for 2 h at 37°C) before the addition of cells. Since induction of CD25 expression was observed within 1 h of stimulation as previously reported (40, 41), we examined incubation periods ranging from 1 to 48 h. CD25 expression was determined by flow cytometry using anti-CD25-FITC (Caltag Laboratories).

The viability of purified and 24-h IL-2–primed (100 U/ml) NK cells after FcγRIIIa-mediated stimulation was determined by quantitation of propidium iodide (PI) uptake in the FACScan® and by direct visual assessment of trypan blue exclusion. Significant changes in cell viability could be detected within 1 h of stimulation with mAb or aggregated IgG (42–44). To determine if FcγRIIIa-mediated stimulation was inducing cell death via apoptosis, in selected experiments cells were fixed, permeabilized, and analyzed for quantitative DNA content. Subdiploid uptake of PI reflects cell death via apoptosis (45). Additionally, we examined stimulated cells for apoptotic morphology (chromatin condensation and nuclear fragmentation) (43) after fixation (2% paraformaldehyde plus 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, followed by post-fixation with 1% osmic acid in cacodylate buffer, pH 7.2). Cells were suspended in 1.5% agar, embedded in Spurr's resin, and the thin sections were viewed in a Phillips CM-12 electron microscope.

Statistical analysis. Differences in ligand binding and mAb binding were analyzed by Student's *t* test. The χ^2 test was used to analyze

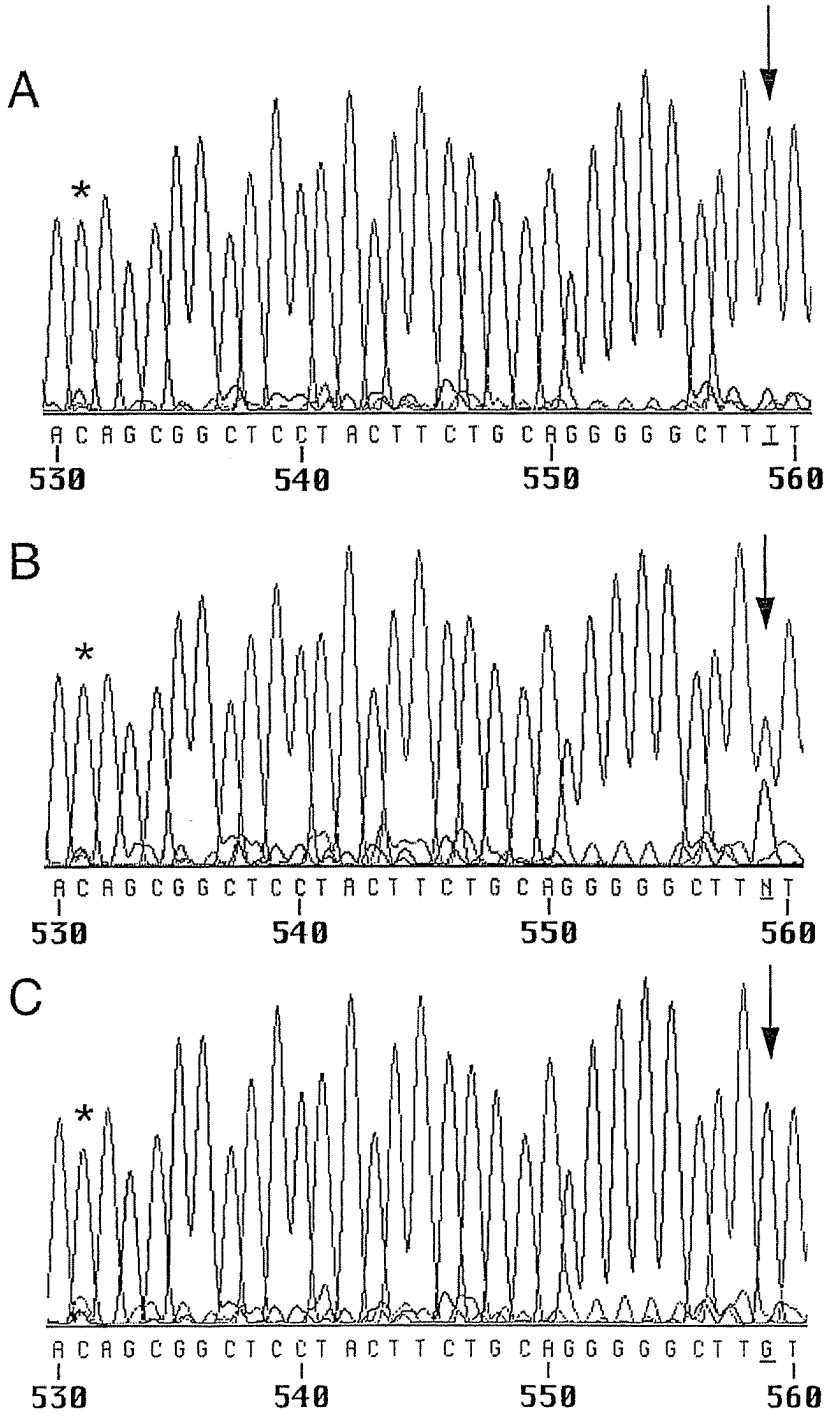

Figure 2. Sequence analysis of a portion of FcγRIIIa cDNA from three normal donors. FcγRIIIa encoding cDNA was prepared from purified MNC and an M13-based dye-primer sequencing strategy was used (see Methods). Donors homozygous for nt 559-T (*A*), homozygous for nt 559-G (*C*), and a donor heterozygous for nt 559-T/G (*B*) are shown. In each tracing, nt 531 (*) is shown to indicate the presence of cDNA encoding the FcγRIIIA gene (C at nt 531) and not the FcγRIIIB gene (which is T at nt 531).

the distribution of FcγRIIIA genotypes (corresponding to 176-V/V, 176-F/F, and 176F/V) in SLE and non-SLE controls. The null hypothesis was rejected at the 95% confidence level ($P < 0.05$).

Results

FcγRIIIA sequence polymorphisms. The recent observation of an FcγRIIIA sequence polymorphism on NK cells which influences ligand binding (24) raised the possibility that this sequence polymorphism might explain previously described differences in NK FcγRIIIa and NK cell function (23). To test this hypothesis we identified several of these normal donors and characterized the nucleotide sequence of their FcγRIIIA. The cell type specific expression of FcγRIIIA and FcγRIIIB in NK cells/mononuclear phagocytes and in neutrophils, respectively, provides a strategy for selective sequencing of cDNAs derived from these two highly homologous genes. Furthermore, within the coding region of FcγRIII, there are 10 nucleotide differences between FcγRIIIA and FcγRIIIB that can be used to confirm the presence of only FcγRIIIA or FcγRIIIB sequence. Using this approach, two normal donors, one with the low binding FcγRIIIa phenotype and one with a high binding FcγRIIIa phenotype (23), were both shown to be T/ T^{230} homozygotes. Interestingly, however, while the low FcγRIIIa phenotype showed no differences from the conventional sequence, the donor characterized phenotypically as high binding FcγRIIIa was heterozygous T/G at nt 559 (Fig. 2). This nucleotide difference, previously mentioned by Ravetch and Perussia (21), raised the possibility that this nonconservative nucleotide polymorphism encoding a phenylalanine to valine at amino acid residue 176^2 in the membrane proximal EC2 of FcγRIIIa might affect ligand binding and receptor function.

Sequence analysis of the entire coding region for MNC FcγRIIIA cDNA from a total of 30 normal donors revealed variation in nt position 559 (T or G). In this group, cDNAs from three individuals contained only G^{559} while six donors contained only T^{559}. The remaining 21 donors were found to contain both T^{559} and G^{559} (Fig. 2). All 30 normal donors were homozygous T at nt 230 and homozygous C at nt 248 (24, 25). There were no other sequence differences throughout the whole FcγRIIIA gene except that two donors were heterozygous at position 249 for a conservative G^{249} to A^{249} substitution, a silent variation at the third position of the codon for serine. These data demonstrate that the sequence variation in the FcγRIIIA gene at nt 559 (amino acid 176) is not a rare mutation, but rather a common polymorphism.

Characterization of CD16 epitopes. To determine if the 176F to V change affects the binding of anti-CD16 mAb which might explain previously reported variations in anti-CD16 mAb reactivity (28, 30, 31, 46, 47), the reactivity of FcγRIIIa on peripheral blood NK cells was characterized using a panel of anti-CD16 mAb. Donors homozygous for 176F or 176V and homozygous for $66L^2$ and $72S^2$ were examined by flow cytometry. Using the well characterized anti-CD16 mAb CLB-Gran1, identical CD16 fluorescence intensities were observed on CD56 positive NK cells from donors of both genotypes (Fig. 3 *A* and Table I). Similar results were evident with six additional anti-CD16 mAbs (Table I) including mAb B73.1 which is affected by the polymorphism at nt 230 (24, 25). mAbs 1D3 and MEM154 showed differential binding to NK cells from donors homozygous for F compared with V. In both instances, these mAb bound well to 176V/V donors but only poorly to 176F/F donors (Fig. 3, *C* and *D*, and Table I). mAb 3G8 showed subtle differences which did not reach statistical significance with our sample size (Fig. 3 *B*). These data indicate that although donors homozygous for either the F or the V alleles express the

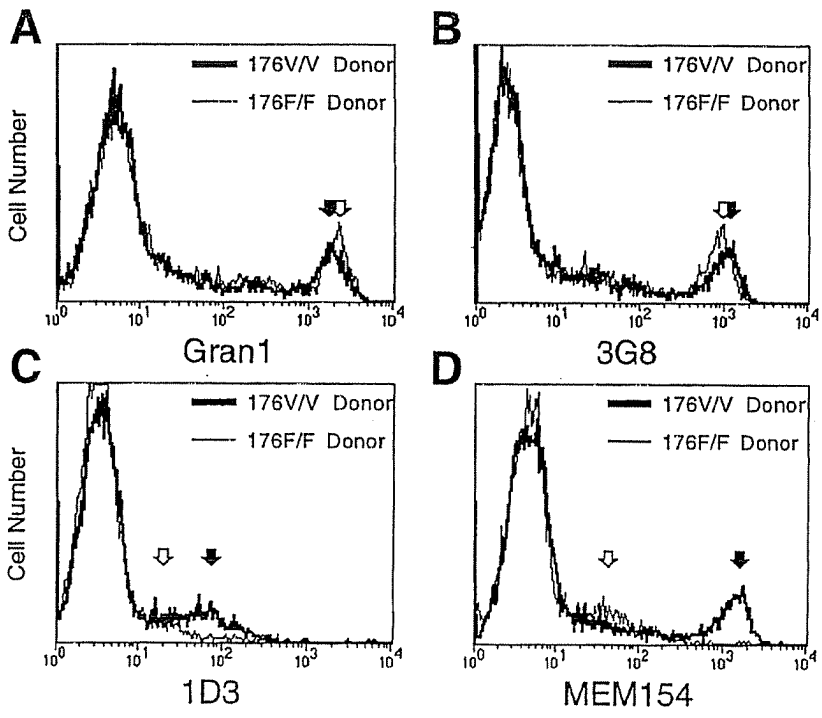

Figure 3. CD16 mAbs MEM154 and 1D3 show differential reactivity with FcγRIIIa-176V and FcγRIIIa-176F alleles. Lymphocytes and NK cells were identified in washed whole blood by characteristic light scatter properties and lack of reactivity with anti-CD14. Anti-CD16 staining on the $CD14^-$ lymphocytes is shown. Blood from donors homozygous for FcγRIIIa-176V/V and FcγRIIIa-176F/F was examined. Identical Gran1 reactivity (*A*) confirms identical receptor density between the two donors. As previously reported, mAb 3G8 binds slightly less to the FcγRIIIa-176F allele than the FcγRIIIa-176V allele (the functional high binding phenotype, see Results) (*B*). mAbs 1D3 (*C*) and MEM154 (*D*) bound well to NK cells from the FcγRIIIa-176V/V donor but reacted poorly with NK cells from the FcγRIIIa-176F/F donor. Data represent a single donor pair out of a total of five different donor pairs examined.

Table I. CD16 Epitopes

mAbs	mAb subclass	Donor type FcγRIIIa-176V/V	FcγRIIIa-176F/F
		n = 5	*n* = 5
CLB-Gran1	mIgG2a	1769±96	1673±191
GRM 1	mIgG2a	1278±108	1356±64
B73.1	mIgG1	1059±15	1113±138
Leu11a	mIgG1	337±25	395±72
3G8	mIgG1	552±69	399±76
30.2	mIgG1	803±50	698±60
214.1	mIgG1	77±17	89±14
135.9	mIgG1	945±58	802±26
MEM154*	mIgG1	1169±126	48±6
ID3‡	mIgM	70±15	34±15

Reactivity of anti-human CD16 mouse mAbs with NK cells expressing the different allelic forms of FcγRIIIa. Data shown are mean channel fluorescence±SEM. ‡$P < 0.003$, FcγRIIIa-176V/V vs. -176F/F; *$P < 0.001$, FcγRIIIa-176V/V vs -176F/F.

same level of CD16 protein on the surface of NK cells, the reactivities for some anti-CD16 mAbs differ, suggesting that the these allelic proteins have different three-dimensional structural characteristics.

We then examined the mAb epitopes expressed on CD16 positive circulating monocytes. This population, typically a small percentage of circulating monocytes (35), was identified by multicolor fluorescence. Because FcγRIIIa expression by monocytes is variable among donors, we sought donors homozygous for 176F or 176V (and homozygous for both 66L and 72S) expressing comparable levels of mAb CLB-Gran1 reactivity on their peripheral blood monocytes. In paired experiments, B73.1 showed identical reactivity while both 1D3 and MEM154 showed less reactivity with the 176 F/F donor (results not shown).

Characterization of ligand binding. A single amino acid change at residue 131 in the membrane proximal domain of FcγRIIa (CD32) results in a nearly 10-fold alteration in quantitative ligand binding of human IgG2 (10–12). To determine if the 176F/V polymorphism in the homologous extracellular domain of FcγRIIIa altered ligand binding, we examined the binding of pooled human IgG and of human IgG myeloma proteins to peripheral blood leukocytes from our homozygous donors. FcγRIIIa has a higher affinity for IgG than FcγRIIa and FcγRIIIb. This higher affinity (reported to be in the range of $1–7 \times 10^7\ M^{-1}$) is less than the affinity of IgG binding to FcγRIa, but is sufficient to allow binding of monomer IgG at physiological concentrations. Binding of pooled human IgG to NK cells was observed in all donors, but the level of binding was substantially different between our homozygous donor groups. Individuals homozygous for both 176V and 66L bound significantly more IgG1 and IgG3 than did donors homozygous for both 176F and 66L (Fig. 4). The difference in binding of IgG1 and IgG3 was observed at both concentrations of IgG (15 and 30 μg/ml) used in these studies. Binding of the myeloma proteins to CD56 positive NK cells was completely blocked by the anti-CD16 mAb CLB-Gran1 (results not shown). A difference in binding of IgG4 (30 μg/ml) was also observed; there was detectable but low binding to donors homozygous for 176V but not 176F. mAb CLB-Gran1 was used to confirm identical levels of CD16 protein on the NK cell surface (Fig. 3). No binding of IgG2 (30 and 15 μg/ml) or IgG4 (15 μg/ml) to NK cells from either donor type was observed.

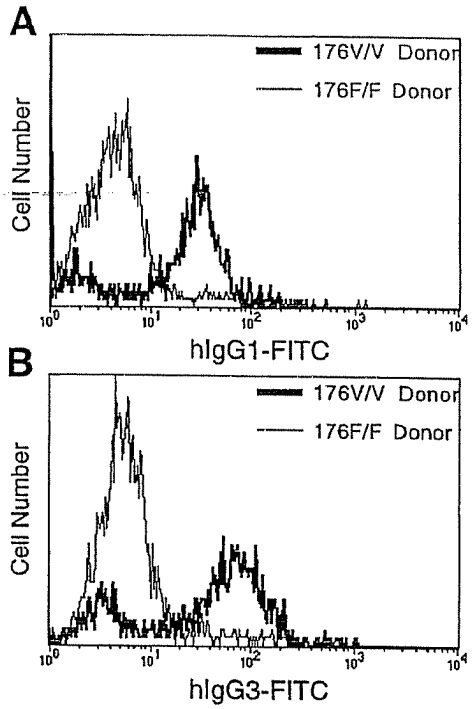

Figure 4. Binding of human IgG myeloma proteins to NK cell FcγRIIIa on donors homozygous for 176V/V and 176F/F. NK cells in washed whole blood were identified by characteristic light scatter properties, by reactivity with anti-CD56, and by lack of reactivity with anti-CD14. Binding of FITC-labeled human IgG1 (*A*) and IgG3 (*B*) (each at 30 μg/ml) to $CD56^+/CD14^-$ cells is shown. NK cells from each donor expressed identical levels of CD16 (using mAb Gran1). Data represent a single donor pair out of a total of five different donor pairs that were examined.

In a number of experiments, we were also able to observe hIgG myeloma protein binding to FcγRIIIa on the small subset of human monocytes expressing CD16. Using preincubation with anti-CD64 mAb 197 to block the high affinity FcγRIa, binding of human IgG to FcγRIIIa could be quantitated. Complete blockade of FcγRIa was confirmed by showing that the binding of mIgG2a (30 μg/ml) was reduced to background autofluorescence levels in the presence of mAb 197 (Fig. 5 *C*). In paired experiments with donor monocytes matched for CLB-Gran1 reactivity and with FcγRIa blocked by mAb 197, 176V homozygous donors bound more hIgG1 than did donors homozygous for 176F (Fig. 5, *A* and *B*). These data document that the nt 559 polymorphism of FcγRIIIA which changes a single amino acid in EC2 results in a change in apparent affinity for ligand binding independent of the cell type in which it is expressed.

Functional implications of the 176F/V polymorphism. To determine if the difference in quantitative binding of ligand to the 176F and 176V alleles results in differences in receptor

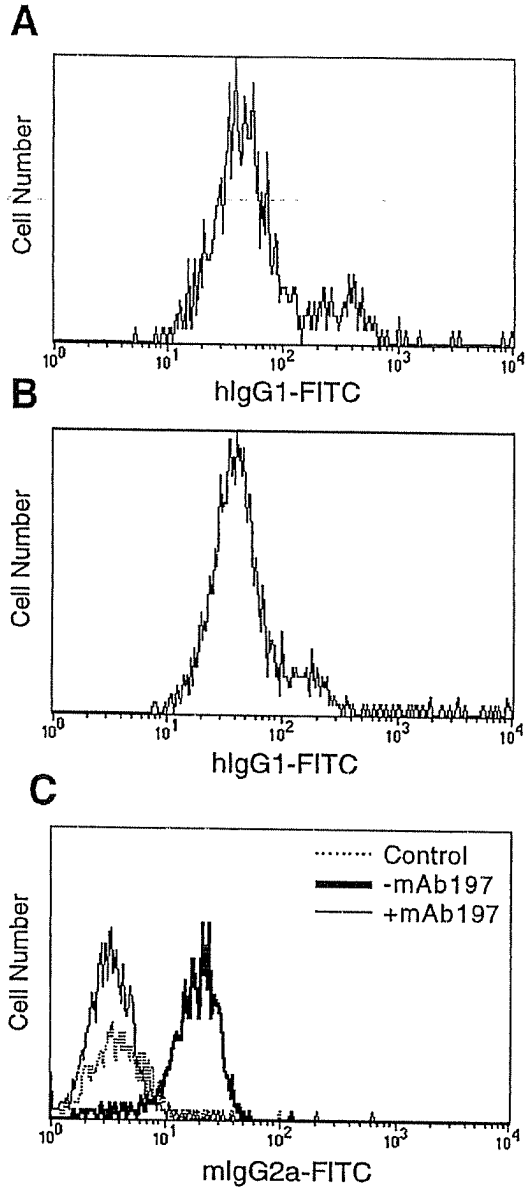

Figure 5. Binding of human IgG1-FITC (30 μg/ml) to peripheral blood monocyte FcγRIIIa on donors homozygous for 176V/V (*A*) and 176F/F (*B*). Monocytes in washed whole blood were identified by characteristic light scatter properties, by reactivity with anti-CD14 (bright and dim), and by lack of reactivity with anti-CD56. The ligand binding site of the high affinity FcγRI was blocked by preincubation with mAb 197. Complete blockade of ligand binding (mIgG2a) is shown (*C*). Monocytes from each donor expressed identical levels of CD16 (using mAb Gran1). Data represent a single donor pair out of a total of four different donor pairs that were examined. Monocytes from the fifth donor pair did not show significant reactivity with anti-CD16. Background fluorescence levels are lower in *C* because of lower detector settings used for the mAb binding panel.

function, we quantitated FcγRIIIa-induced NK cell activation. Cross-linking of FcγRIIIa results in an immediate rise in $[Ca^{2+}]_i$ (48, 49). When purified NK cells from 176F and 176V homozygous donors were stimulated with the anti-CD16 mAb 3G8 and $F(ab')_2$ GAM, a brisk rise in $[Ca^{2+}]_i$ was observed. The magnitude of this rise in $[Ca^{2+}]_i$ in the homozygous donors was indistinguishable (466 and 516 nM in the 176F and 176V homozygous donors, respectively; Fig. 6). When aggregated human IgG was used as the stimulus, a rapid rise in $[Ca^{2+}]_i$ was also observed in the homozygous donors, but the magnitude of the rise in NK cells from the 176V homozygote donor was more than threefold greater than the rise observed in NK cells from the 176F homozygote donor (56 and 189 nM in the 176F and 176V homozygous donors, respectively; Fig. 6).

To examine the impact of the 176F/V polymorphism on integrated cell functions, upregulation of surface CD25 (IL-2 receptor) on IL-2–treated NK cells after engagement of FcγRIIIa was assessed (40, 41). Stimulation with mAb 3G8 and $F(ab')_2$ GAM induced rapid upregulation of expression of CD25 (IL-2R) on the surface of purified NK cells. Using purified NK cells from homozygous donors, engagement and cross-linking of CD16 with IgG aggregates also resulted in rapid upregulation of CD25 expression. However, donors homozygous for 176V showed significantly higher levels of CD25 expression relative to donors homozygous for 176F (Fig. 7).

FcγRIIIa on NK cells is important in regulating NK cell survival through receptor-mediated activation-induced cell death (42–44). Both anti-CD16 mAb plus GAM cross-linker and IgG aggregates decreased NK cell survival quite rapidly. However, while comparable levels of cell survival were apparent after anti-CD16 mAb stimulation, there was a marked difference between 176F/F and 176V/V donors in the degree of NK cell death after stimulation with IgG aggregates (Fig. 8). Nuclear fragmentation and chromatin condensation, characteristic of apoptosis and assessed by transmission electron microscopy, was observed in NK cells stimulated via FcγRIIIa (with cross-linked mAb or aggregated IgG) (results not shown). In addition, quantitative PI staining of fixed and permeabilized cells demonstrated a distinct population of apoptotic cells with subdiploid DNA content in aggregated IgG stimulated but not control cells (Fig. 9).

Characterization of the 176F/V polymorphism in donors with disparate antibody-dependent cellular cytotoxicity (ADCC) activity. Because of the clearly defined differences in FcγRIIIa-induced function in our homozygotes, we considered the possibility that the differences in quantitative ADCC by NK cells of different donors, previously described by Vance (23), might reflect the 176F/V polymorphism. This possibility was reinforced by the original observation that the difference in ligand binding among these donors was much greater than the difference in mAb 3G8 binding. Accordingly, we made cDNA from MNC preparations from six previously characterized individuals. Four low binding FcγRIIIa phenotype donors were homozygous for T^{559} (176F), for T^{230} (66L), and for C^{248} (72S) while the two other donors, characterized phenotypically as high binding FcγRIIIa, were G/T^{559} heterozygous and homozygous for T^{230} (66L) and for C^{248} (72S) indicating that NK cell ADCC is influenced by the 176F/V polymorphism (23). These data demonstrate the functional importance of variation of nt 559 in an independently phenotyped group of donors.

Association of the 176F allele with autoimmune disease. We have shown previously that the low binding allele of FcγRIIa (131H) is associated with SLE and nephritis in African-American patients (16). To determine if skewing of the normal 176F/V allelic system might also be associated with SLE, we developed a genotyping assay based on allele-specific PCR.

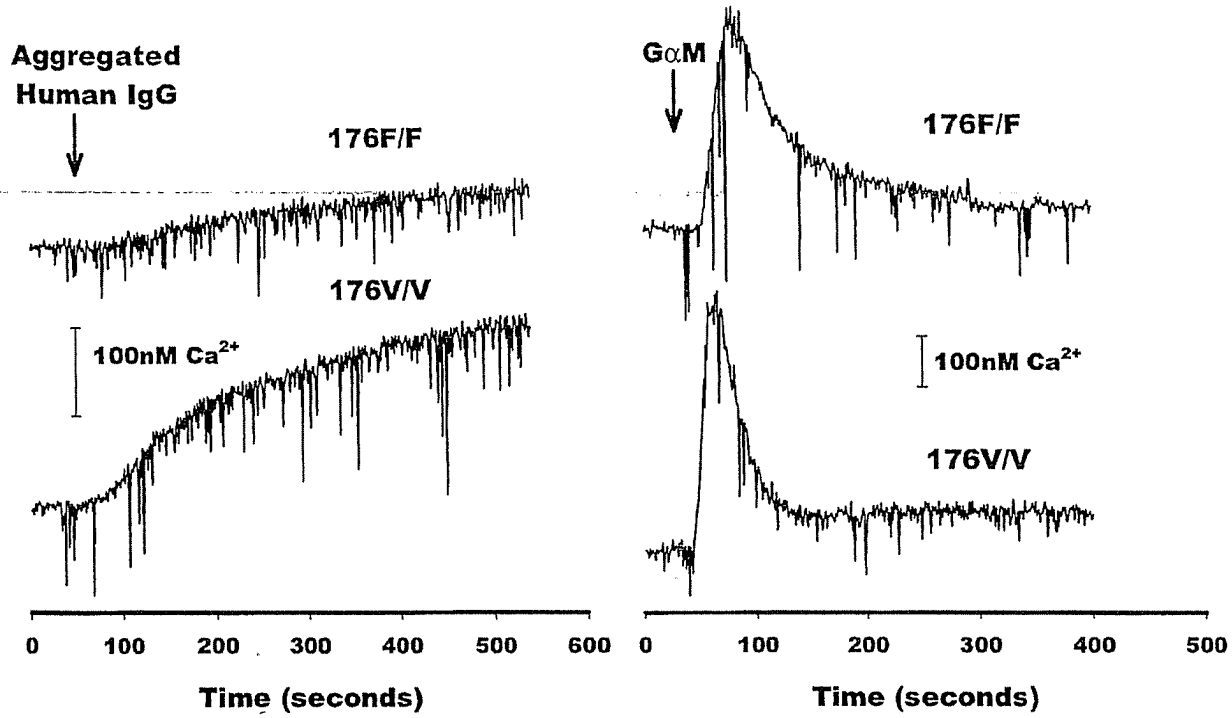

Figure 6. Aggregated hIgG and anti-CD16 mAb 3G8 induced rise in intracellular Ca^{2+} levels in purified NK cells from donors homozygous for FcγRIIIa-176V/V and FcγRIIIa-176F/F. Cells were isolated using a magnetic negative depletion strategy (Miltenyi NK Cell Isolation Kit) and were > 90% CD56 and CD16 positive after isolation. NK cells were loaded with indo-1-AM and stimulated at 60 s with either 60 μg/ml aggregated hIgG (*left*) or were prelabeled with mAb 3G8 $F(ab')_2$ and then stimulated with $F(ab')_2$ GAM at 60 s (*right*). In all experiments, the rise in $[Ca^{2+}]$ induced with aggregated hIgG is much larger in the FcγRIIIa-176V/V donor (mean peak $[Ca^{2+}]$ (nM) above baseline in 176V/V and 176F/F donors; 199±21 and 67±11, respectively, $P < 0.01$, $n = 3$). Indistinguishable changes in $[Ca^{2+}]$ in both donors were induced by cross-linked mAb 3G8 (mean peak $[Ca^{2+}]$ (nM) above baseline in 176V/V and 176F/F donors; 555±51 and 526±137, respectively, $P > 0.05$, $n = 3$). A representative experiment of three (with different donor pairs) is shown.

The fidelity of this assay was established using a genomic template from our initial group of FcγRIIIa sequenced normal donors ($n = 30$) and confirmed by the sequencing of selected SLE patients ($n = 38$) and additional normal donors ($n = 11$). Using this assay, we genotyped a population of 200 ethnically diverse patients with documented SLE and a cohort of 113 ethnically diverse normal individuals. There was a significant skewing in the distribution of the three genotypes (3 × 2 contingency table, $\chi^2 = 9.87$, $P < 0.01$; Table II) and in the allelic frequency (2 × 2 contingency table, $\chi^2 = 6.13$, $P < 0.015$; Table II) between the two groups. In the SLE patients, there was an increase in homozygosity for 176F; 44% of the 200 SLE patients but only 23% in the 112 non-SLE control subjects were 176F homozygous. In contrast, only 4% of the 79 SLE patients with nephritis were 176V/V homozygotes compared with 15% of the 121 nonrenal SLE patients (15% in normal controls). These results suggest that the presence of the FcγRIIIa 176F allele is a significant risk factor for development of SLE, especially with nephritis.

Discussion

The recent observation of an FcγRIIIA sequence polymorphism on NK cells which influences ligand binding (24) raised the possibility that this sequence polymorphism might explain previously described differences in NK FcγRIIIa and NK cell function (23). To test this hypothesis we identified several of these normal donors and characterized the nucleotide sequence of their FcγRIIIA. Contrary to our expectation, these donors were monomorphic at nt 230 and nt 248 but polymorphic at nt 559. This polymorphism predicts an F to V substitution in position 176 of EC2 of FcγRIIIA, the domain which is critical for ligand binding. Normal donors, homozygous for F and for V at position 176 and homozygous for L at position 66 and for S at position 72 in all cases, were characterized for ligand binding and for FcγRIIIa function. NK cells and monocytes from donors with 176V/V bound more IgG1 and IgG3 than the corresponding cells from 176F/F donors. FcγRIIIa-176V/V elicited a larger flux in $[Ca^{2+}]_i$, a greater degree of cell activation, and a more pronounced program of activation-induced cell death than FcγRIIIa-176F/F. FcγRIIIa-176F/F individuals were overrepresented in a population of 200 SLE patients while FcγRIIIa-176V/V individuals were underrepresented among patients with nephritis. These data, coupled with other observations (14–20), suggest an important role for FcγR polymorphisms in human disease.

The absence of the V allele in the phenotypic low binding donors and its presence in the high binding donors strongly

Table II. Distribution of FcγRIIIa Alleles in SLE Patients and Non-SLE Controls

	SLE patients	Non-SLE controls
	n = 200	*n = 113*
Genotype*		
No. of subjects (% of group)		
176F/F	87 (44%)	29 (26%)
176F/V	92 (46%)	69 (61%)
176V/V	21 (10%)	15 (13%)
Allelic frequency‡		
176F	0.67	0.56
176V	0.33	0.44

A PCR-based genotyping assay (using genomic DNA) was developed using allele specific primers (see Results). 200 ethnically diverse patients with documented SLE (33) and 113 ethnically diverse normal volunteers were genotyped for FcγRIIIa alleles [nt 559G (176V) and/or nt 559T (176F)]. Allele and gene frequencies are shown. *SLE patients vs. normal controls; 3×2 contingency table, $\chi^2 = 9.87$, $P < 0.01$; ‡SLE patients vs. normal controls; 2×2 contingency table, $\chi^2 = 6.13$, $P < 0.015$.

suggest that this polymorphism explains the difference in NK FcγRIIIa originally described by Vance and colleagues (23). In that cohort, the tendency toward lower reactivity with mAb 3G8 among low binders is consistent with our data demonstrating the same subtle trend (Fig. 3 *B* and Table I). Most importantly, in the Vance study the ratio of IgG binding to mAb 3G8 reactivity clearly shows reduced ligand binding, even when mAb 3G8 is used to define receptor number. Less clear, however, is the relationship between the 176F/V polymorphism and the NK FcγRIIIa described in several patients with recurrent infections (26). Both of those patients showed markedly reduced reactivity with mAb B73.1. Based on our data that the B73.1 epitope is not influenced by position 176 and other data indicating that 66R/R donors have markedly reduced levels of B73.1 reactivity (24), we anticipate that these individuals with recurrent infections have some difference in NK cell FcγRIIIa other than variation at position 176. Indeed, the polymorphism at amino acid position 66 may contribute to this difference (26). Quantitatively, however, the approximate twofold increase in IgG1 binding reported for donors with 66R or 66H compared with 66L parallels the magnitude of the difference in IgG binding we have seen with 176V compared with 176F. Although differences in ligand binding can influence susceptibility to infection (14, 15), this functional similarity between the polymorphisms at 66 and 176 and the prevalence of the 176F/V polymorphism makes this mechanism an unlikely basis for the rare patients described to date (26). However, the similarity in ligand binding raises the interesting question of whether the difference in ligand binding described for donors varying in position 66 might be explained by allelic association with position 176. To date, in more than 80 normal donors, we have identified only two individuals who are heterozygous T/G at nt 230 (66L/66R), one individual who is heterozygous T/A at nt 230 (66L/66H), and one individual who is homozygous at nt 230 for the uncommon A allele (66H). Therefore, we have been unable to test the hypothesis that 66R and 66H occur in association with 176V and that 176V may determine the ligand binding phenotype.

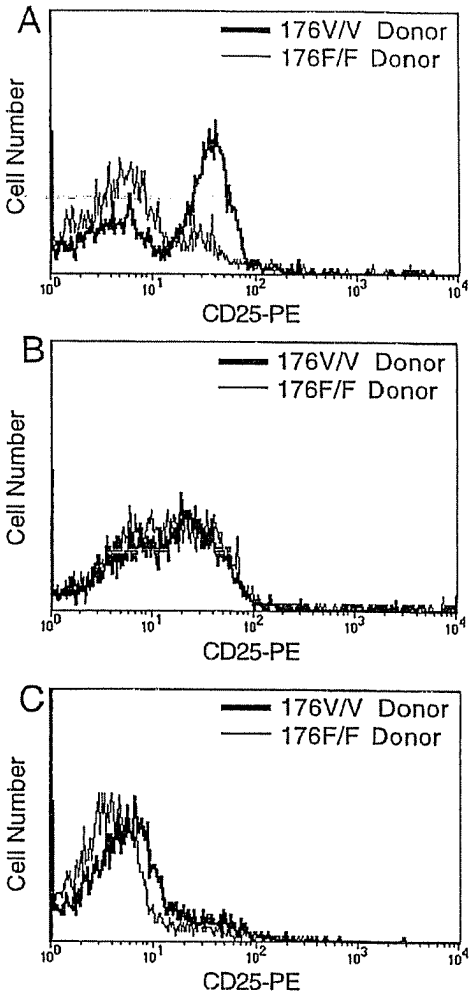

Figure 7. Aggregated hIgG and anti-CD16 mAb 3G8 induced upregulation of CD25 expression on purified NK cells from donors homozygous for FcγRIIIa-176V/V and FcγRIIIa-176F/F. Cells were isolated using a magnetic negative depletion strategy (Miltenyi NK Cell Isolation Kit) and were > 90% CD56 and CD16 positive after isolation. CD25 expression was determined after cells were primed overnight with IL-2 (100 U/ml) and a with subsequent 2-h stimulation with either 60 μg/ml aggregated hIgG (*A*), anti-CD16 mAb 3G8 (2.5 μg/ml) on GAM-coated plates (*B*), or buffer as control (*C*). Data illustrated represent a single donor pair out of four different donor pairs that were examined. The mean percent CD25 positive cells after aggregated hIgG stimulation was 66.4±8.8 for 176V/V donors and 34.9±19.4 for 176F/F donors ($P < 0.03$). Stimulation of NK cells from 176V/V and 176F/F donors with anti-CD16 mAb consistently produced a change in mean percent CD25 positive cells relative to control but there was no significant difference in the level of induction between donors ($P > 0.05$).

There are several interesting implications of the FcγRIIIa 176F/V polymorphism. Recent data have suggested that FcγR expressed on macrophages may play an important role in the regulation of serum IgG levels. Initial observations in the FcγRII knockout mouse demonstrated an impact on total IgG levels, but since the entire FcγRII gene with its various splice

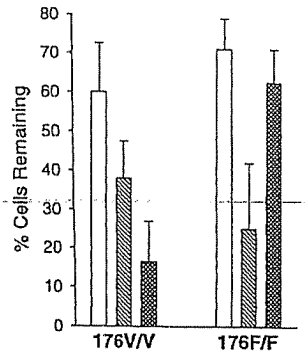

Figure 8. Aggregated hIgG and anti-CD16 mAb 3G8 induced cell death of purified NK cells from donors homozygous for FcγRIIIa-176V/V and FcγRIIIa-176F/F. Cells were isolated using a magnetic negative depletion strategy (Miltenyi NK Cell Isolation Kit) and were > 90% CD56 and CD16 positive after isolation. Cell viability was determined in the presence of trypan blue by cell counting by two independent observers. NK cells were stimulated for 24 h with IL-2 (100 U/ml) and then subsequently with buffer as control (*open bars*), anti-CD16 mAb 3G8 (2.5 μg/ml) on GAM-coated plates (*striped bars*), or 60 μg/ml aggregated hIgG (*hatched bars*). The mean number of cells remaining (±SEM, $n = 4$) for 176V/V donors after aggregated IgG stimulation was significantly less than for 176F/F donors ($P < 0.05$); the two groups were not different in control and anti-CD16 stimulation conditions.

isoforms was disrupted, the relative roles of FcγRIIb1 expressed on B cells as opposed to FcγRIIb2 expressed on macrophages could not be determined (50). Somewhat surprisingly, the naturally occurring disruption of the expression of the FcγRIIb2 isoform expressed on macrophages of NOD mice is strongly associated with upregulation of both IgG1 and IgG2b serum levels despite relatively normal expression of the B cell specific FcγRIIb1 (51). The possibility that macrophage FcγR may be playing an important role in the regulation of IgG levels is further underscored by the observation in humans that different alleles of FcγRIIa are associated with different serum levels of IgG2 (14). This observation is particularly important because in humans, FcγRIIa is expressed on macrophages but not on B cells. Furthermore, this observation emphasizes that alleles with different capacities to bind human IgG2, not just presence or absence of receptor, are associated with different levels of IgG. Thus, it seems reasonable to extrapolate to the prediction that the FcγRIIIa 176F/V alleles may influence the level of IgG1 and IgG3. How they influence specific responses to vaccination and net effective humoral immunity remains to be determined.

Of course the implications of the FcγRIIIa 176F/V polymorphism extend beyond the regulation of serum IgG levels. Soluble FcγRIIIa is clearly present in the circulation (52–54). In a number of systems, soluble receptor can influence the level of B cell activation presumably through binding surface immunoglobulin (55, 56). Since FcγRIIIa binds ligand with higher affinity than FcγRIIIb which is unable to bind ligand in monomeric form, FcγRIIIa may play a particularly important role in mediating these effects. FcγRIIIa may also play a critical role in the first-dose cytokine-release syndrome seen with some therapeutic monoclonal antibodies (57). Furthermore, our earlier studies in a primate model of immune complex handling demonstrated an essential role for FcγRIIIa (58, 59), and more recent observations in mice with targeted disruption of murine FcγRIII also support an important role in immune complex–mediated triggering of inflammatory reactions (60). Since each of these effects is dependent on binding of IgG, the potential for FcγRIIIa 176F/V alleles to influence the biologic potential of both receptor and ligand is clearly evident.

To directly test this potential in human biology in vivo, we investigated the possibility that FcγRIIIa 176F/V alleles might be abnormally represented in patients with SLE, a prototypic immune complex disease. A role for abnormal FcγR function in SLE has been described (61), and the skewing of FcγRIIa alleles in SLE has supported the hypothesis that the FcγRIIa allele with a low binding phenotype for human IgG2 would be overrepresented in SLE (16, 17). In most immune complexes, however, autoantibodies are not of the IgG2 isotype but rather of the IgG1 and IgG3 isotypes. Thus, one might anticipate an overrepresentation of 176F and an underrepresentation of 176V in immune complex disease. In our study of 200 ethnically diverse SLE patients, this skewing was very apparent (Table II). Indeed, in patients with SLE and nephritis, the homozygous 176V was underrepresented by more than fourfold compared with those without nephritis. We recognize that these observations need confirmation in large independent populations, that further stratification by clinical phenotype and ethnicity may be insightful, and that studies of multiplex families will be informative. We also recognize that this association may result from linkage to a different gene at another locus. However, the biology of this polymorphism, its relevance to the pathophysiology of SLE, and the coincidence of FcγRIIIa's chromosomal location with a region of high interest in the microsatellite-based scanning of the genome in SLE patients (62, 63), all make FcγRIIIa a likely gene for SLE disease risk.

Based on the biology of the FcγRIIIa-176F/V polymor-

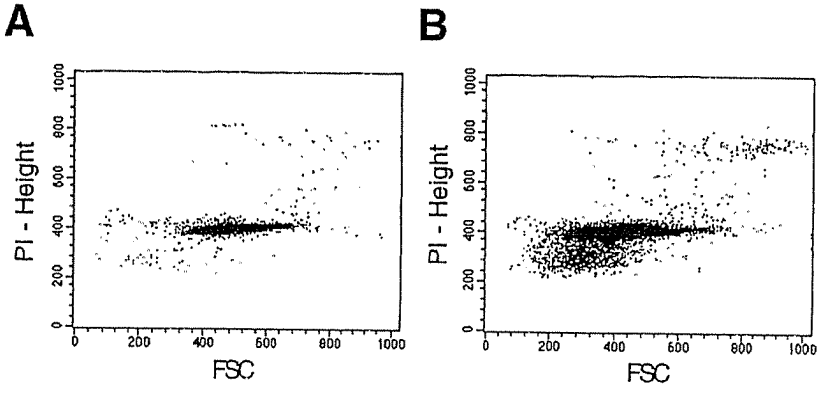

Figure 9. Stimulation of purified IL-2 primed NK cells with hIgG aggregates induces the appearance of a subdiploid population of cells characteristic of apoptotic cells. After overnight incubation with IL-2 (100 U/ml), cells were treated with buffer (*A*) or IgG aggregates (*B*) for 1 h, 37°C. Cells were then fixed, permeabilized, and stained for quantitative DNA content with PI (see Methods). To allow discrimination of cell doublets, PI width and PI area were collected and cell fragments were excluded through analysis of SSC (side light scatter) and FSC (forward light scatter).

phism, one can imagine that it could influence many antibody-mediated responses involving IgG1 and IgG3. Since FcγRIIIa is expressed on NK cells, mononuclear phagocytes, and renal mesangial cells, host defense against viral, bacterial, and other pathogens could be affected. Antibody-mediated immune surveillance could be altered as well as the interaction with immune complexes. Furthermore, the therapeutic response to intravenous gammaglobulin might vary in accordance with the FcγRIIIa-176F/V polymorphism. Indeed, characterization of Fcγ receptor genotypes, in conjunction with other properties of the humoral immune response such as antibody subclass and complement status, may provide essential insights into vaccine effectiveness and disease risk.

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